# VOLATILES IN BIOLOGICAL SPECIMENS BY DUAL HEADSPACE GAS CHROMATOGRAPHY (HS/GC)

## PRINCIPLE

Volatile compounds are analyzed in biological fluids by gas chromatography using N-propyl alcohol as the internal standard. Water containing an internal standard is added simultaneously with the biological sample as it is sampled using an automatic diluter. It is then sealed in a headspace sample vial prior to analysis. Volatile sample components are extracted from the non-volatile sample components by heating, pressurizing the vial and then sampling from the equilibrated gas phase above the sample phase. One milliliter of this gas phase mixture is injected onto a column, which splits into 2 different gas chromatographic columns. The volatile compounds are separated based on their respective molecular weights and polarities and detected with a flame ionization detector. The identification of ethanol and other volatile compounds is made by comparing the relative retention times of the unknown to the retention time of an internal standard. The ratio of sample peak area to internal standard peak area is compared to the calibration curve to provide a quantitation of volatile compounds in the sample.

By using 2 different columns that cause the volatiles to separate in different but known ways, a more specific identification is possible. The possibility of an interfering or co-eluting peak is also considerably reduced since it is unlikely to elute on both columns at the same retention time.

## BACKGROUND AND PHARMOACOKINETIC INFORMATION

Ethanol is the most widely used central nervous system (CNS) depressant. Sometimes methanol, isopropanol, and other volatile compounds are substituted for ethanol, thereby causing other toxic effects in the user. The following method is used to analyze biological fluids for ethanol; the attached appendices summarize the modifications necessary to analyze biological fluids for methanol, isopropanol, and acetone. Effects of these volatile compounds can be found in the corresponding section of the Toxicology Training Program.

## SPECIMEN

1. BLOOD: Use blood specimens collected in gray top evacuated tubes (containing 100 mg sodium fluoride and 20 mg potassium oxalate). Blood specimens collected in other containers may be analyzed. The optimum sample size is 2 mL or greater. Specimens containing less than 2 mL may be analyzed.
2. URINE: Use urine specimens collected in urine collection bottles. Urine specimens collected in other containers may be analyzed. The optimum

specimen size is 2 mL or greater. Specimens containing less than 2 mL may be analyzed.

3. Keep specimens at 0°C to 8°C until analyzed; bring the specimens to room temperature before analysis.
4. If a specimen contains an insufficient sample for analysis, the submitting agency is notified on the final report.

## SAFETY PRECAUTIONS

1. Wear safety glasses, laboratory coats, and gloves when handling reagents, samples, and controls.
2. Sample preparation procedures should be performed under a biosafety hood.
3. Processed headspace vials are both heated and pressurized; take caution when removing from the autosampler, as they may be burst upon impact.

## REAGENTS AND SUPPLIES

1. N-propyl alcohol (high purity)[1,2,3]
2. 200 proof (Pure Ethyl Alcohol) Dehydrated U.S.P [1,2,3]
3. Commercially prepared Ethanol Calibrators (0.02, 0.04, 0.08, 0.20, 0.40 g/dL)
4. Acetone (high purity) [1,2,3]
5. Isopropanol (high purity) [1,2,3]
6. Methanol (high purity) [1,2,3]
7. 1L and 2L volumetric flask
8. 1 mL, 5 mL, and 10 mL volumetric pipettes
9. 10 mL headspace vials
10. 20 mm butyl septa
11. 20 mm aluminum seals
12. Headspace vial racks
13. Automatic diluter

[1] Highly Flammable – avoid heat or flame.
[2] Irritant – avoid contact with skin, eyes, etc. – avoid inhaling fumes.
[3] Health – may be harmful if swallowed, inhaled, or absorbed through skin.

14. Tube rocker
15. Homogenizer
16. Gas Chromatograph with dual flame ionization detector and autosampler
17. Chemstation™ software

## STANDARDS PREPARATION

Label all reagents with reagent name, tracking number, preparation date, preparer's initials, and expiration date. Store in a ground glass stoppered reagent bottle. Record the standard reagent information in LIMS.

1. Ethanol Working Standard Preparation:

   After opening the ethanol standard ampoule, transfer the solution to its individual amber glass vial and cap with a screw cap. Standards are good for 2 weeks from the time they are opened. Store vials in refrigerator at 0°C to 8°C when not in use.

   For the 0.79 g/dL Working Standard: Allow 200 proof ethanol to reach room temperature (~25°C). Add 1 mL of ethanol to 100 mL de-ionized water and mix well. Transfer solution to an glass container and cap with a screw cap. This standard is good for 6 months from the date of preparation. Store in refrigerator at 0°C to 8°C when not in use.

2. N-propyl Alcohol Internal Standard (IS) Preparation:

   Add 200 µL N-propyl Alcohol to 2 L ultrapure water and mix well. Add 250 mg of sodium fluoride to prevent mold growth. Label flask with internal standard name, lot number, analyst's initials, and tracking number. Record the internal standard reagent information on the reagent log. Internal standard solution is good for 1 year from the date of preparation and can be stored at room temperature.

## CONTROL PREPARATION

All controls must be prepared from a separate stock source than the standards. Separate vendors and/or lot numbers are sufficient to fill this requirement.

1. Whole Blood Reference Control:

   Prepared by manufacturer. Store in the refrigerator at 0°C to 8°C; expiration is 30 days from the time it is opened.

2. Serum Interference Control (contains ethanol, methanol, acetone, isopropanol):

   Prepare according to manufacturer instructions. Store in the refrigerator at 0°C to 8°C; expiration is 30 days from the time it is prepared.

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## CALIBRATION PROCEDURE

1. Quantitation analysis:

   Calibrators consist of six levels: 0.01, 0.04, 0.08, 0.20, 0.40, 0.79 g/dL.

2. Calibrate the gas chromatograph at the beginning of every batch of analysis with calibrators in order of increasing concentration. A minimum of 3 calibrators is needed to construct the calibration curve.

3. Values must fall within ± 6% of the target values before new calibrator lots can be introduced.

## QUALITY CONTROL PROCEDURE

1. Quality controls samples consist of:

   1.1. Whole Blood Reference Control: Store in the refrigerator at 0°C to 8°C; expiration is 30 days from the time it is opened.

   1.2. Serum Interference Control (contains ethanol, methanol, acetone, isopropanol): Store in the refrigerator at 0°C to 8°C; expiration is 30 days from the time it is prepared.

2. Analyze one of each control in each batch of analysis.

3. Values must fall within ± 6% of the target values before a new Whole Blood Reference Control lot can be introduced.

4. Record Whole Blood Reference Control results in LIMS.

5. Each of the 4 peaks in the Serum Interference Control must be present before the new lot can be introduced.

6. The instrument calculations are verified annually as described in SOP-030 Instrument Calculation Verifications.

## SAMPLE PREPARATION PROCEDURE

1. Bring calibrators, controls, and samples to room temperature.

2. Mix blood samples for at least 3 minutes on a tube rocker. Clotted samples should be homogenized prior to sampling and this step should be indicated in the report.

3. Determine the number of headspace vials needed and label the vials in the following sequence order:

Calibrators (in increasing order)

Internal Standard Blank

Whole Blood Control

Serum Reference Control

≤ 20 sample injections

Check standard

≤ 20 sample injections

Check standard, etc.

4. Use the automatic diluter to dispense 1.4 mL of internal standard mixture and 0.05 mL of calibrators, controls, and samples into a headspace vial.
5. An internal standard blank is prepared by placing the internal standard mix into a sample vial.
6. Cap and seal the specimen by crimping a headspace cap onto the vial.
7. Enter the analytical sequence by using the Chemstation™ software.
8. Load the vials onto the autosampler tray according to the analytical sequence and analyze the samples with the Chemstation™ Ethanol program. Typical HS/GC conditions are included in Appendix C.

## CALCULATIONS

1. Calibrators:

   A calibration curve is derived by comparing the ratios of the calibrator ethanol peak areas to their respective internal standard peak areas. The ratio of sample peak area to internal standard peak area is compared to the calibration curve to provide a quantitation of any volatile compounds in the sample. The Chemstation™ software calculates a "least squares" line.

   1.1. The calibrator coefficient ($r^2$) must be ≥ 0.99.

   1.2. Check standards must fall within the limits of the calibration curve. Values must be within ± 6% of the target value. If a check calibrator falls outside this range, all positive samples not bracketed by acceptable check standards must be reanalyzed.

   1.3. The blank result must be lower than the lowest calibrator.

2. Controls:

   2.1. The result of the Whole Blood Reference Control must be within ± 6% of the target value. If a Whole Blood Reference Control falls outside this range, all positive samples must be reanalyzed.

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3. Samples:

   3.1. The dual HS/GC generates 2 values for each sample analyzed; duplicate samples will generate 4 values. All values are truncated after the 3rd decimal before calculation. The lowest and the highest of the 4 values must fall within ± 5% of their mean for any sample with results ≥ 0.08 g/dL. For sample with values < 0.08 g/dL, the difference from the mean should be no more than ±0.004 g/dL. If these requirements are met, the lowest value is reported to the 2nd decimal place.

## REPORTING RESULTS

1. Results obtained are recorded in LIMS, following the guidelines outlined in the LIMS User Manual.
2. Any exceptions to the criteria stated within this procedure and all data affected by the exceptions must be clearly documented, in LIMS and the case file, and need to be submitted for the technical review.

## LIMITATIONS OF PROCEDURE

1. The lower method detection limit is 0.002 g/dL. The upper method detection limit is 15.8 g/dL (detector limit reached).
2. The lower limit of quantitation for the method is 0.005 g/dL. The upper limit of quantitation for the method is 6.87 g/dL.
3. For DUI cases, the reporting limit ranges from 0.01-0.79 g/dL. For autopsy specimens, the reporting limit ranges from 0.02-0.79 g/dL.

## PROCEDURE NOTES

1. For quantitative batches, samples must be analyzed in duplicate. Any sample that overloads the detector will be diluted and reanalyzed.
2. If a sample contains acetone, isopropanol, or methanol, reanalyze the sample in duplicate using the method described in Appendix A.
3. Any sample with large extraneous peaks should be evaluated for the presence of other volatile compounds and/or interferences. For some common interferences, see Appendix A.
4. Autopsy samples are screened for volatiles using the method described in Appendix B.

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## REFERENCES

1. Agilent Chemstation help index, topic Calibration curves.
2. Roger L. Firor and Chin-Kai Meng, “Static Headspace Blood Alcohol Analysis with the G1888 Network Headspace Sampler”.
3. Restek Corp.’s “A Technical Guide for Static Headspace Analysis Using GC”.
4. Matthew T. Barnhill, Jr., Donald Herbert, and David J. Wells, Jr., “Comparison of Hospital Laboratory Serum Alcohol Levels Obtained by Enzymatic Method with Whole Blood Levels Forensically Determined by Gas Chromatography” Journal of Analytical Toxicology Vol. 31 2007
5. Butala, Steven J.M., PhD, “Estimation of Bureau of Toxicology Laboratory Error for Blood Alcohol Utilizing Direct Injection GC/FID: Addendum: Estimation of Bureau of Toxicology Laboratory Error for Blood Alcohol Utilizing Headspace GC/FID” Bureau of Environmental Chemistry, Utah Department of Health.

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## APPENDIX A:

## OTHER VOLATILES IN BIOLOGICAL SPECIMENS BY HS/GC

The analysis for Acetone, Isopropanol, and Methanol is conducted as per the Ethanol Procedure with the following exceptions.

### STANDARDS PREPARATION

1. Volatiles (Acetone, Isopropanol, Methanol) Working Standard Preparation:

   After opening the volatiles standard ampoule, transfer the solution to its individual amber glass vial and cap with a screw cap. Standards are good for 2 weeks from the time they are opened. Store vials in refrigerator at 0°C to 8°C when not in use.

### CALIBRATION PROCEDURE

1. Quantitation analysis:

   1.1. Calibrators consist of three levels: 0.05, 0.10 and 0.40 g/dL.

   1.2. Calibrate the gas chromatograph at the beginning of every batch of analysis with calibrators in order of increasing concentration. A minimum of 3 calibrators is needed to construct the calibration curve.

   1.3. Values must fall within ± 10% of the target values before new calibrator lots can be introduced.

### QUALITY CONTROL PROCEDURE

1. The quality control sample consists of a Serum Reference Control (contains ethanol, methanol, acetone, isopropanol). Store in the refrigerator at 0°C to 8°C; expiration is 30 days from the time it is opened.
2. Analyze one control in each batch of analysis.
3. Values must fall within ± 10% of the target values before a new Serum Reference Control lot can be introduced.
4. Serum Control Results are reported in LIMS.

### CALCULATIONS

1. The result of the Serum Interference Control must be within ± 10% of the target value. If the Serum Control falls outside this range, all positive

samples must be reanalyzed for the compounds which are outside of acceptable criteria.

2. Check standards must fall within ± 10% of the target value. If a check calibrator falls outside this range, all positive samples not bracketed by acceptable check standards must be reanalyzed.

## PROCEDURE NOTES

1. Comparing relative retention times (RRT) of an unknown peak and the internal standard peak allows identification of the unknown peak. The following table summarizes the RRTs for the common volatile compounds on the two different columns of the HS/GC.

| Compound | RRT db-alc1 | RRT db-alc2 |
|---|---|---|
| Methanol | 0.493 | 0.464 |
| Ethanol | 0.616 | 0.584 |
| Acetone | 0.898 | 0.642 |
| Isopropyl alcohol | 0.746 | 0.683 |
| N-propyl alcohol (IS) | 1.00 | 1.00 |

2. The following table provides a summary of RRTs of other volatile compounds on the two different columns of the HS/GC. With only one exception (chloroform), all tested interferences caused conflicts in only 1 column and may be compensated for by using data from the other column.

   The minor peaks for chloroform are the only known interferences for both columns, and this occurs only when the chloroform has broken down. When fresh chloroform is present, no minor peaks were observed. If the main chloroform peak is present then it *may* contribute to any ethanol seen.

| **Elution Order DB-Alc1** | | | | **Elution Order DB-Alc2** | | |
|---|---|---|---|---|---|---|
| **#** | **Compound** | **RRT db-alc1** | | **#** | **Compound** | **RRT db-alc2** |
| 1 | Methanol | 0.493 | | 1 | Acetaldehyde | 0.435 |
| 2 | Formaldehyde | 0.501/0.833 | | 2 | Methanol | 0.464 |
| 3 | Acetaldehyde | 0.542 | | 3 | Formaldehyde | 0.474 / 0.580 |
| 4 | Ethanol | 0.616 | | 4 | Ethyl ether | 0.516 |

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| | | | | | |
|---|---|---|---|---|---|
| 5 | Ethyl ether | 0.736 | 5 | Ethanol | 0.584 |
| 6 | Isopropyl alcohol | 0.746 | 6 | Acetone | 0.642 |
| 7 | Methylene chloride | 0.826 | 7 | Hexane | 0.666 /**m** |
| 8 | Acetone | 0.898 | 8 | Isopropyl alcohol | 0.683 |
| 9 | Acetonitrile | 0.907 | 9 | Methylene chloride | 0.697 |
| 10 | N-propyl alcohol (IS) | 1.000 | 10 | Acetonitrile | 0.794 |
| 11 | Hexane | 1.146 /**m** | 11 | N-propyl alcohol (IS) | 1.00 |
| 12 | Chloroform | 1.285 /**m** | 12 | Ethyl acetate | 1.05 /**m** |
| 13 | Iso-butyl alcohol | 1.550 | 13 | Heptane | 1.152 |
| 14 | Ethyl acetate | 1.680 | 14 | Chloroform | 1.183 /**m** |
| 15 | n-Butyl alcohol | 1.985 /**m** | 15 | Iso-butyl alcohol | 1.590 |
| 16 | Heptane | 2.280 | 16 | n-Butyl alcohol | 2.115 /**m** |
| 17 | Iso-amyl Alcohol | 3.530 /**m** | 17 | Toluene | 2.880 /**m** |
| 18 | Toluene | 4.215 | 18 | Iso-amyl Alcohol | 3.760 / 3.830 /**m** |
| 19 | m-Xylene | 9.750+ | 19 | m-Xylene | 6.824 |

**m = multiple minor peaks**

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## APPENDIX B:

## AUTOPSY VOLATILES SCREEN

Autopsy specimens are screened for volatiles as per the Ethanol Procedure with the following exceptions.

### CALIBRATION

1. Calibrators consist of three levels: 0.02, 0.08, 0.40 g/dL. Samples ≥0.02 g/dL are verified with a quantitation analysis.

### QUALITY CONTROL PROCEDURE

1. The quality control sample consists of a Serum Reference Control (contains ethanol, methanol, acetone, isopropanol). Store in the refrigerator at 0°C to 8°C; expiration is 30 days from the time it is opened.
2. Analyze one control in each batch of analysis.
3. Values must fall within ± 10% of the target values before a new Serum Reference Control lot can be introduced.
4. Record Serum Reference Control results on QC Database.

### SAMPLE ANALYSIS

1. For autopsy volatiles screens, samples may be analyzed in singlet; positive samples are analyzed in duplicate in a quantitation batch.

**Prepared By:** Signature: _____ ____/____/_____
Title: _____

**Analyst Review:** Signature: _____ ____/____/_____
Title: _____

**Approval:** Signature: _____ ____/____/_____
Title: _____

# VOLATILES IN BIOLOGICAL SPECIMENS BY DUAL HEADSPACE GAS CHROMATOGRAPHY (HS/GC)

## VOLATILES BY HS/GC REVISIONS

| Date | Rev. # | Revision | |
|---|---|---|---|
| | | **Former procedure** | **New procedure** |
| 3/24/06 | | No requirement was provided. | Check standards used must be within the range of the calibration curve. |
| | | Preparation instructions for 0.79 ethanol working standard was for 2L and only 2 week expiration date. | Preparation instructions for 0.79 ethanol was changed to smaller volume (1L) and 6 month expiration date. |
| | | When introducing new lots of calibrators or whole blood control, values must agree within 10% of old lots. | When introducing new lots of calibrators or whole blood control, values must agree within 10% of target values. |
| 6/1/06 | 060106 | No HSGC parameters were included. | Typical HSGC parameters included as Appendix C. |
| | | When running quantitation batch for methanol/isopropanol/acetone, no QC data was tracked. | When running quantitation batch for methanol/isopropanol/acetone, QC data must be tracked on database. |
| 11/17/06 | 111706 | Typos/grammatical changes | No change in procedure. |
| 01/01/08 | Rev. 03 | When introducing new lots of calibrators or whole blood control, values must agree within 10% of target values. | When introducing new lots of calibrators or whole blood control, values must agree within 6% of target values. |
| | | For Ethanol: Whole Blood Control must be within 5% of the target value. | For Ethanol: Whole Blood Control must be within 6% of the target value. (This will be consistent with the statistical study where confidence levels were evaluated.) |
| | | For Other Volatiles: Serum Control must be within 5% of the target value. | For Other Volatiles: Serum Control must be within 10% of the target value. (This will be consistent with manufacturer recommendations until a statistical study can be performed.) |
| | | For Ethanol: Low calibrator was set at 0.02 g/dL. | For Ethanol: Low calibrator was changed to 0.01 g/dL. (This will be consistent with our reporting cut-off levels) |
| | | For Other Volatiles: Calibrators were prepared in house at levels of 0.039, 0.079, and 0.197. | For Other Volatiles: Calibrators have been changed to commercial preparation at levels of 0.05, 0.10, and 0.40. |
| | | For Other Volatiles: Lower reporting cut-off level was 0.02 g/dL. | For Other Volatiles: Lower reporting cut-off was changed to 0.05 g/dL. (This will be consistent with our lowest calibrator.) |
| | | Revisions tracked in separate document. | Revisions added as last page within the analytical method. |

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| Date | Rev. # | Revision | |
|---|---|---|---|
| | | **Former procedure** | **New procedure** |
| 09/09/08 | Rev.04 | | Added Section to Quality Control addressing annual instrument calculation verifications |
| | | Reporting statements and were listed under “reporting results” Control results were reported to QA Database | Reporting Results, Control results, and Stds prep sections were updated to reflect use of the new LIMS system. |
| | | Calibration curve had 0.79 calibrator as optional | Calibration curve updated to include 0.79 calibrator as a standard part of the procedure, consistent with LIMS. |